REMARKS

Rejection of claims under 35 U.S.C. 112:

Claims 7-14 and 18 have been rejected under §112, second paragraph. The claims have been amended to obviate the rejection.

Claim 7 has been amended to comply with Applicants' telephone interview with the Examiner. The unclear language has been removed and the original claim 7 has been separated into three independent claims and new claims have been added consistent with our discussion.

Dependent claims 10 and 11 have been amended by removing the confusing language as pointed out in the Action. Claims 12-14 and 18 have been cancelled to obviate the rejection.

A §132 Declaration has been submitted with this Amendment and Response as technical background to the specification.

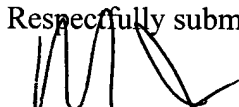Rejection of claims under 35 U.S.C. 102/103:

Claims 7-14 and 18 have been rejected under §102(b) as being anticipated by Wagner *et al.* Applicants have amended the claims to be consistent with the telephone interview.

The original independent claim 7 has been separated into 3 independent claims in order to clarify the intent. New dependent claims have been added that are based upon the originally filed dependent claims. The rejection based upon Wagner *et al.* is believed to be overcome.

The reference to Stassen *et al.* is also believed to be overcome by the amendments.

The Examiner's objections and rejections are believed to be overcome by this response to the Office Action. In view of Applicants' amendments and discussion, it is submitted that independent claims 7, 19 and 24 are allowable and therefore dependent claims 8-11 and 18-23, and 25-28, which depend either directly or indirectly from the independent claims, should be allowable as well. Applicants respectfully request an early notice to such effect.

Respectfully submitted,

Mark K. Johnson   Reg. No. 35,909
P.O. Box 510644
New Berlin, WI 53151-0644
262.821.5690

I hereby certify that this correspondence is being deposited with the United States Postal Service as EXPRESS MAIL - POST OFFICE TO ADDRESSEE, in an envelope addressed to: Commissioner for Patents, Washington, DC 20231 on _5/3/02_

EJ342652805US

Mark K. Johnson   Express Mail #

4

The rate at which a disulfide bond is reduced depends on the acidity of the thiols (pK_a) which make up the disulfide bond. The lower the pK_a of the thiol, the more rapidly the disulfide bond would be predicted to be reduced [1-4]. The disulfide containing monomers that were employed in the study were derived from 3-mercaptopropionic acid (pK_a = 10.8)[3], cysteine (pK_a =8.3)[1], and 5-thio-2-nitrobenzoic acid (pK_a =5) [5, 6]. Based on the pK_a values, the predicted rate of disulfide reduction is 3-mercaptopropionic acid < cysteine < 5-thio-2-nitrobenzoic acid.

The synthesis of 12 polymers (six containing disulfide bonds, and six controls) was initiated as previously described. (Figure 1) The polymers derived from the imidate ester system (a 3-mercaptopropionic acid derivative) proved to be the most straightforward system initially tested (compounds 5, 6, 11, and 12). Analysis of the polymers by a mass spectrometer (both prior to and after degradation with 2-mercaptoethanol, PE SCIEX API 150 EX Turbo Ionspray Mass Spectrometer) indicated the correct mass for the subunit. Purification of the polymers was accomplished by size exclusion chromatography under high salt concentrations (to minimize charge interactions with the column packing). Ammonium acetate was found to be the best salt in the purification process because it is volatile, and therefore removed upon lyophilization of the sample. It proved to be important in subsequent studies that the salts be removed from the sample in order to avoid salt effects with the polymer – pDNA complex (discussed below). Ammonium bicarbonate may also be utilized as the salt in the column purification (ammonium bicarbonate is more volatile than ammonium acetate), however, disulfide bond cleavage was observed in some samples (especially for the 5,5'-dithiobis(2-nitrobenzoic acid) systems), due to the high pH of the solution. The size exclusion columns were standardized with poly-L-lysine molecular weight standards, in order to establish molecular weight distributions for the synthesized polymers.

Polymers derived from the amidation of a carboxylic acid were also synthesized. The analysis (mass spectroscopy) for the polymers derived from 5,5'-dithiobis(2-nitrobenzoic acid) or N-Boc cystine and triethylenetetramine (compounds 7 and 9) indicated that some acylation of the secondary amine had taken place in the reaction (utilizing a carbodiimide and N-hydroxysuccinimide in the amidation reaction), causing polymer chain branching. For example, compound 9 was estimated to have approximately 20 % of secondary amine acylation. We have investigated using the PyBOP phosphonium based reagent in the presence of hydroxybenzotriazole under concentrated reaction conditions for the activation of the carboxylic acid (for the amidation reaction). The resulting polymers were essentially free from secondary amine acylation and additionally, contained polymer in the 40,000 to 60,000 molecular weight range based on analysis by size exclusion chromatography.

Deprotection of the N-Boc groups for the cystine derived polymers (and control polymers, compounds 3, 4, 9, and 10), was conducted under acidic conditions (trifluoroacetic acid). Trifluoroethanol (and hexafluoroisopropanol) have been useful, particularly in peptide synthesis, for disrupting peptide interactions leading to better synthetic yields and facilitating removal of protecting groups. Addition of small percentages of trifluoroethanol (<2.5%) to the deprotection reaction facilitated the BOC removal from the polymer, resulting in ultimately greater than 95% deprotection. All polymers were purified either by dialysis or by size exclusion chromatography (both methods under high concentrations of ammonium acetate) in order to remove small molecular weight material and isolate polymer in the 5,000 to 15,000 molecular weight range.
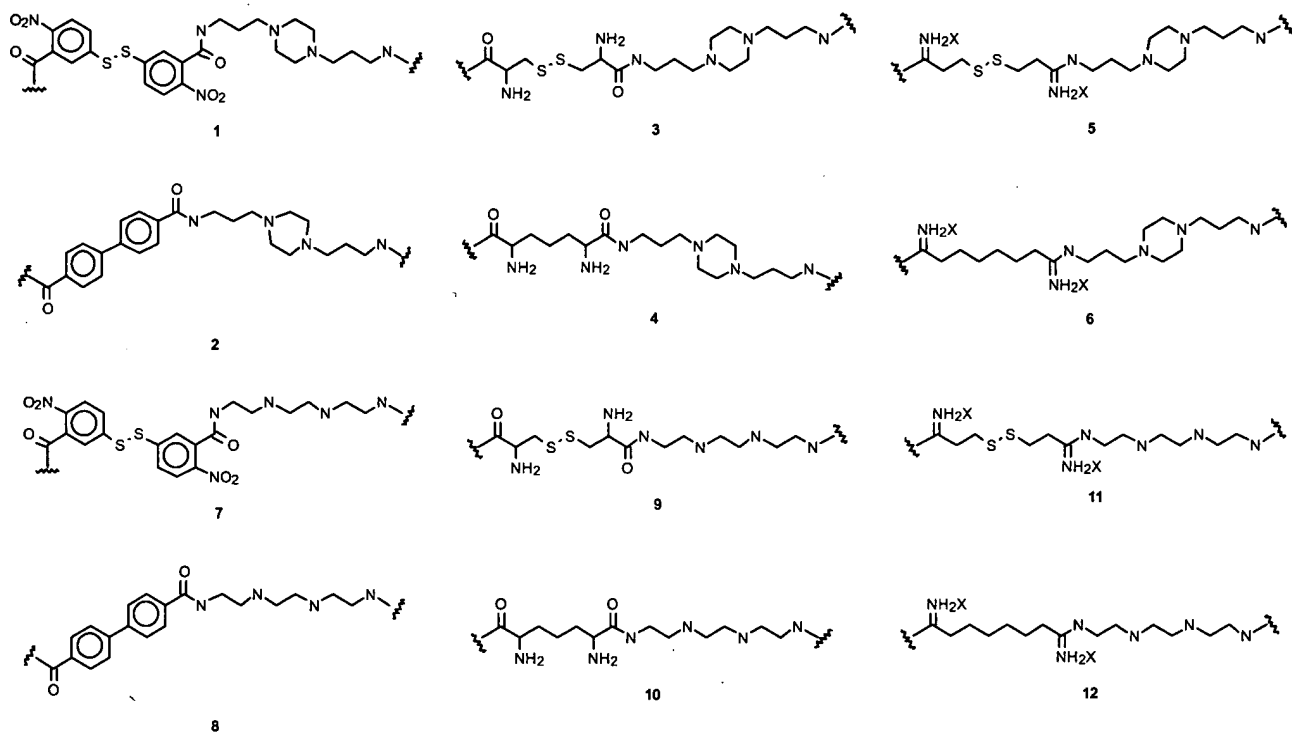
Figure 1. Structures of Polymers Constructed to Evaluate Disulfide Lability

Polymer in vitro testing:

Polymers 1 and 7 were analyzed for polymer degradation in the presence of 0, 0.1, 0.5, and 1 mM glutathione by monitoring the absorbance at 412 nm (the λmax of the thiolate from 5,5'-dithiobis(2-nitrobenzoic acid). Cleavage was not observed in the absence of glutathione, however, rapid degradation was observed for all samples in the presence of glutathione (t ½ 1.1 – 3.6 min). Other polymers could not be evaluated by this method due to the lack of spectroscopic thiolate produced upon degradation. Therefore the polymers were analyzed after complexation with pDNA. Polymers 1 and 7 were similarly analyzed for degradation following particle formation in order to evaluate the lability of the disulfide bond comparatively for the different systems.

Recent studies conducted at Mirus have looked at the effective condensation of pDNA by a variety of polycations such as poly-L-lysine and polyethylenimine. In effect, a titration method employing fluorescence quenching has been developed, in order to establish the effective mass per charge of a polycation based on the polymers condensation of pDNA [7]. Briefly, 10 μg of rhodamine labeled pDNA (approximately 100 base pairs per label) is taken up in 1 mL of 25 mM HEPES at pH 7.4. Aliquots of the polycation are added to the DNA solution until no further fluorescence quenching is noted, indicating the amount of polymer needed to form a 1:1 charge complex. Given the molecular weight per phosphate group in the pDNA (330), the effective molecular weight per charge for the polycations can be calculated. By this method, the effective charge for poly-L-lysine is the same as the theoretical mass per charge (208 for the hydrobromide salt). Other polycations with more closely packed amines deviate from the theoretical mass per charge. For example, polyethylenimine, a widely used nonviral vector has a theoretical mass per charge of 45 based on the polymer repeating unit,

however, the effective charge for the branched version was determined to be 83 and the linear version deviates even more with an effective mass per charge of 132. This effect can be explained by the pKa's of the polyamine, a higher amine density affects the ability of neighboring amines to protonate and become charged (charge – charge repulsion). Additionally, the molecular structure of a polymer has an effect on the ability of the system to effectively condense DNA. It was thought that the titration method would be an effective method for the evaluation of our synthetic polymers, minimizing effects from incomplete amine deprotection, amine acylation, and variations in salt form, thereby normalizing the polymers for complex formation with pDNA.

The actual mass per charge from the titration study was utilized in the formulation of polymer – pDNA complexes of 0.7:1, 2:1, and 3:1. The complexes were tested *in vitro* for lability against glutathione. The results indicated that the half life of the particles indeed followed the expected trend based on thiol pKa, with the polymers derived from 5,5'-dithiobis(2-nitrobenzoic acid) being more labile than the cystine derived polymers which were in turn much more labile than the dimethyl 3,3'-dithiobispropionimidate dihydrochloride derived polymers. (Table 1) The dimethyl 3,3'-dithiobispropionimidate dihydrochloride derived polymers in fact showed no more decondensation than any of the control polymers, even in the presence of serum (as an anionic challenge) at the highest level of glutathione tested (Table 1, under 10% after 24 hrs). Difficulties were encountered with the 5,5'-dithiobis(2-nitrobenzoic acid) and 4,4'-biphenyldicarboxylic acid systems due to solubility of the polymer, poor pDNA condensation, and aggregation in the samples. The t 1/2 values show the general trend 0.7:1<2:1<3:1, where the lower the charge ratio of the particle the shorter the half-life (all supporting data not shown). The large half-life determined for the 3:1 charge ratio complex with compound 9 could have to do in part with the amount of charge remaining after degradation. It is known that small polyamines such as spermine condense pDNA. In the study, serum was added to provide an anionic challenge (from the high concentration of negatively charged albumin in serum) to the polycation if no decondensation was observed within 30 minutes. Additional studies investigated complex half-life in the presence of 0, 0.1 and 0.5 mM glutathione as proposed. Very little difference in t 1/2 values were observed at the 0.5 mM glutathione level. However, for both the 0, and 0.1 mM glutathione level, very little polymer cleavage was noted.

Table 1. Polymer – pDNA Complex Half-life's In The Presence of Glutathione

Compound	Polymer Type	MolWt.	Glutathione (mM)	t ½min (0.7:1)	t ½min (2:1)	t ½min (3:1)
1	5,5'-Dithiobis(2-nitrobenzoic acid)	8-12 K	1	0.2	0.5	0.5
2	5,5'-Dithiobis(2-nitrobenzoic acid)	8-12 K	1	X[a]	X[a]	X[a]
3	Cystine	8-12 K	1	5.5	7.5	7.5
4	Cystine control	8-12 K	1	5,900[b,c]	60,000[b,c]	48,000[b,c]
5	3-Mercaptopropionic acid	8-12 K	1	X[b,c,d]	10,000[b,c]	9,900[b,c]
6	3-Mercaptopropionic acid control	8-12 K	1	X[b,c,d]	13,000[b,c]	7,300[b,c]
7	5,5'-Dithiobis(2-nitrobenzoic acid)	8-12 K	1	X[a]	X[a]	X[a]
8	5,5'-Dithiobis(2-nitrobenzoic acid) control	8-12 K	1	X[a]	X[a]	X[a]
9	Cystine	8-12 K	1	6.5[c]	38[c]	720[c]
10	Cystine control	8-12 K	1	X[b,c,d]	X[b,c,d]	X[b,c,d]

11	3-Mercaptopropionic acid	8-12 K	1	$72,000^{b,c}$	$11,000^{b,c}$	$9,900^{b,c}$
12	3-Mercaptopropionic acid control	8-12 K	1	$23,000^{b,c}$	$18,000^{b,c}$	$34,000^{b,c}$

a) particles aggregate. b) t ½ min calculated from the % of decondensation of the polymer – pDNA complex observed after 24 hrs., assuming no change in kinetics. c) in the presence of 5% serum. d) no decondensation observed.

The results from the complex stability tests confirmed that the polymer – pDNA complexes from the 5,5'-dithiobis(2-nitrobenzoic acid) or cystine systems were susceptible to glutathione reduction, leading to destruction of the complex. An additional factor, polymer length, was next considered. Although more stable than the 5,5'-dithiobis(2-nitrobenzoic acid) polymers, the cystine derived polymers showed good lability and better solubility profiles, and were therefore chosen for the initial assessment into the affect of the polymer size on *in vitro* characteristics of the polymer – pDNA complex and on the *in vivo* transfection efficiency in muscle. The polymers were synthesized as previously described and purified by size exclusion chromatography. (Figure 2) Several molecular weight ranges (based on poly-L-lysine standards) were collected for evaluation (a 4-7 K, b 8-12 K, c 13-16 K, d 17-21 K, e 22-27 K, f 28-33 K, g >34 K).
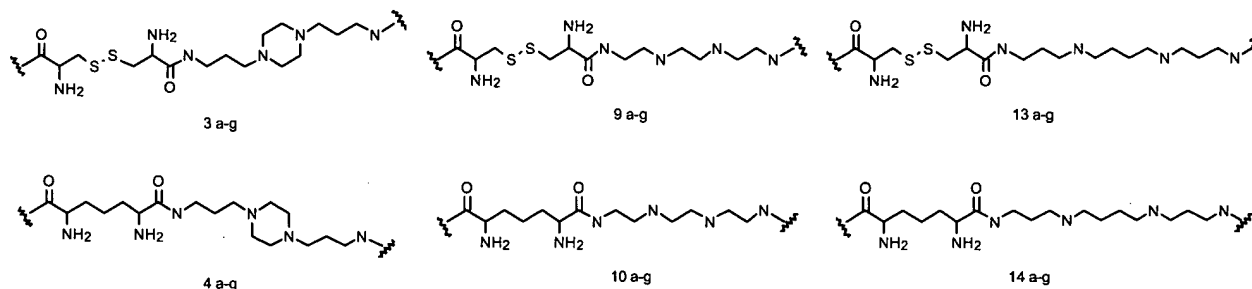

Figure 2. **Structures of Polymers Constructed to Evaluate Polymer Molecular Weight Affects**

The polymer molecular weight fractions were evaluated as before for their effective charge in DNA condensation. Some variation in effective charge was observed for different molecular weight fractions, however, the variation observed was small, with no discernable pattern. Additionally, analysis of the various molecular weight fractions of the polymers did not elucidate any structural variation, suggesting that the effective charge differences were due to experimental variability. Additionally, small differences in the final salt concentration within each sample could have an effect in the condensation strength of the sample. The determined effective charge was utilized in complex formation in order to evaluate changes in lability (upon exposure to glutathione) due to polymer molecular weight. (Table 2) In addition to the previously noted trend based on charge ratio (t 1/2, 0.7:1<2:1<3:1), the results indicated that the polymer molecular weight also influences particle stability in the presence of glutathione. The particle half-life generally decreased as the molecular weight of the polymer increased for complexes reduced with 1 mM glutathione. This observation is not yet fully understood, and the trend was not consistent for polymer – pDNA complexes reduced with lower amounts of glutathione (0.5 mM glutathione). The 0.5 mM glutathione testing indicated increased particle half-life as compared to 1 mM glutathione reducing conditions. No significant lability was observed at 0 and 0.1 mM glutathione levels (even in the presence of serum).

Table 2. Polymer – pDNA Complex Half-lives In the Presence of Glutathione

Compound	MolWt.	Glutathione (mM)	t ½min (0.7:1)	t ½min (2:1)	t ½min (3:1)
3a	4-7 K	1	X[a]	7.0	6.8
3c	13-16 K	1	7.4	6.5	6.9
3f	28-33 K	1	3.9	5.2	5.8
3g	>34 K	1	3.5	4.5	5.6
3a	4-7 K	0.5	X[a]	6.5	8.6
3c	13-16 K	0.5	3.8	9.3	10.1
3f	28-33 K	0.5	2.2	6.7	7.5
3g	>34 K	0.5	3.1	7.1	8.1

a) poor condensation

References:

1   Bulaj, G., Kortemme, T. and Goldenberg, D. P. (1998) Biochemistry **37**, 8965-8972
2   Keire, D. A., Guo, W. and Rabenstein, D. L. (1992) Magnetic Resonance in Chemistry **30**, 746-753
3   Keire, D. A., Strauss, E., Guo, W., Noszal, B. and Rabenstein, D. L. (1992) Journal of Organic Chemistry **57**, 123-127
4   Szajewski, R. P., Whitesides, G.M. (1980) Journal of the American Chemical Society **102**, 2011
5   Whitesides, G. M., Lilburn, J., Szajewski, R.P. (1977) Journal of Organic Chemistry **42**, 332
6   Ellman, G. L. (1959) *Arch. Biochem. Biophys* **82**, 70
7   Trubetskoy, V. S., Slattum, P. M., Hagstrom, J. E., Wolff, J. A. and Budker, V. G. (1999) Anal Biochem **267**, 309-13

Example 1: Synthesis of Cysteine-Terminal Tat Peptide (Tat-Cys).

Peptide syntheses were performed using standard solid phase peptide techniques using FMOC chemistry. A cysteine was added to the amino terminus of Tat to allow for conjugation through the thiol group to make the peptide Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys (Tat-Cys).

Example 2: Synthesis of noncleavably linked (irreversible covalent) Tat-Cys and fluorescein through a thioether bond.

To a solution of succinimidyl-4-(N-maleimidomethyl) cyclohexane-carboxylate (SMCC from Pierce) 1.0 mg in 0.1 mL dimethylformamide was added 1.2 mg (1 eq) of 4'-(aminomethyl)fluorescein. After two hours, this solution was added to a 1 mL aqueous solution of 8.4 mg Tat-Cys (1 eq). The solution was buffered to pH 8 by the addition of potassium carbonate. This solution was used for transport studies without further purification.

Example 3: Synthesis of Unactivated (non-labile) Disulfide Linked Lissamine Dimer (Dilissamine Cystamine)

To a solution of cystamine dihydrochloride (10 mg) in water (1mL) was added diisopropylethylamine (15 mL, 2 eq). To this was added lissamine chloride (Rhodamine B sulfonyl chloride, Molecular Probes) 77 mg (3 eq) in 5 mL of methanol. The solution was stirred for 1 hour and then chromatographed by reverse-phase HPLC using an Aquasil C-18 column using a gradient from 100% 0.1% trifluoroacetic acid in water to 100% 0.1% triflouroacetic acid in acetonitrile. The fraction containing the product was determined by mass spectroscopy. The molecular weight of compound is 1234, which was detected in positive ion mode. The concentration of the product-containing fraction was determined by the absorbance of the solution at 588 nm (e=88,000 M-1cm-1).

Example 4: Attachment of Lissamine to Tat-Cys by an Unactivated Disulfide

To a solution of Tat-Cys (100 mg) in 100 mL water was added dilissamine cystamine (41 mg, 1 eq). The pH of the solution was adjusted to 7-8 by the addition of potassium carbonate.

Example 5: Synthesis of Activated (labile) Disulfide-Containing Lissamine Adduct (Lissamine 4-aminophenyl disulfide)

To a solution of 10 mg of lissamine chloride (Rhodamine B sulfonyl chloride, Molecular Probes) in 0.2 mL dimethylformamide was added over five minutes ten 10 mL aliquots of 4-aminophenyl disulfide (2 mg, 0.5 eq) and diisopropylethylamine (3 mL, 1 eq). Two hours after final addition of disulfide the solution was diluted into 2 mL of acetonitrile and chromatographed by reverse-phase HPLC using an Aquasil C-18 column applying a gradient from 20% acetonitrile and 80% water containing 0.1% trifluoroacetic acid to 100% 0.1% triflouroacetic acid in acetonitrile. We were unable to isolate the lissamine dimer, but were able to isolate the product of monoaddition. The fraction containing the monoaddition product was determined by mass spectroscopy. The molecular weight of compound is 789, which was detected in positive ion mode. The concentration of the product-containing fraction was determined by the absorbance of the solution at 588 nm (e=88,000 M-1cm-1)

Example 6: Attachment of Lissamine to Tat-Cys by an Activated Disulfide
To a solution of Tat-Cys (100 mg) in 100 mL water was added Lissamine 4-aminophenyl disulfide (26 mg, 1 eq). The pH of the solution was adjusted to 7-8 by the addition of potassium carbonate.

Example 7: Synthesis of Activated (labile) Disulfide Fluorescein Dimer (Difluorescein 4-aminophenyl disulfide)
To a solution of 20 mg of fluorescein isothiocyanate in 0.5 mL dimethylformamide was added 4-aminophenyl disulfide (4 mg, 0.33 eq) in 100 mL dimethylformamide and diisopropylethylamine (3 mL, 0.33 eq). After two hours, the solution was diluted into 2 mL of water that was brought to pH 8 with potassium carbonate. This aqueous solution was filtered and chromatographed by reverse-phase HPLC using an Aquasil C-18 column applying a gradient from 100% water containing 0.1% trifluoroacetic acid to 100% 0.1% triflouroacetic acid in acetonitrile. The fraction containing the product was determined by mass spectroscopy. The molecular weight of compound is 1025, which was detected in negative ion mode. The concentration of the product-containing fraction was determined by the absorbance of the solution at 494 nm (e = 75,000 M-1cm-1)

Example 8: Measurement of the Reduction of Unactivated (Non-labile) Disulfide (dilissamine cystamine).
To a solution containing 0.44 mM dilissamine cystamine and 100 mM sodium phosphate pH 7.5 was added glutathione to a concentration of 250 mM. The solution was irradiated with 555 nm light and the fluorescence of the solution was measured at 585 nm. The amount of time required to reach half maximum fluorescence was 2000-2400 sec.

Example 9: Measurement of the Reduction of Activated (labile) Disulfide (difluorescein 4-aminophenyl disulfide).
To a solution containing 0.44 mM fluorescein 4-aminophenyl disulfide and 100 mM sodium phosphate pH 7.5 was added glutathione to a concentration of 250 mM. The solution was irradiated with 495 nm light and the fluorescence of the solution was measured at 520 nm. The amount of time required to reach half maximum fluorescence was 30-50 sec.

Example 10: Analysis of delivery to cells by TAT peptide.
Grow HeLa cells on glass coverslips by incubating at 4°C in Delbecco's Modified Eagle's Media (DMEM) supplemented with 50 μg TAT peptide-fluorophore chimera (pulse). At this temperature, endocytosis is believed to be completely inhibited. Incubate the cells for two hours at 4°C and then wash with DMEM to remove external TAT-fluorophore. Remove the media and then either process cells for fluorescence microscopy or incubate three more hours at 4°C with DMEM with media changes every hour (chase). The cells that are chased are then processed for fluorescence microscopy. Cells processed for fluorescence microscopy are washed 3x in phosphate-buffered saline (PBS), fixed in PBS + 4% formaldehyde for 20 min, washed 3x in PBS, and coverslips are mounted on slides.

The presence of fluorophore was detected by confocal microscopy (Zeiss LSM 510). In the case of irreversible covalent thioether linkage between TAT and fluorophore, fluorescence was detected inside of the cell after the initial two hour incubation. Subsequent incubation of the cells with fluorophore-free media (chase) resulted in cells with no internalized fluorophore. Similarly, TAT-fluorophore adducts linked through an unactivated disulfide cystamine bond also had initial internalization that disappeared upon incubation with chase solutions. For the activated disulfide 4-aminophenyl disulfide, fluorescence was detected inside of the cell after the initial two hour incubation. In contrast to the other attachments between flourophore and TAT, a chase of the fluorophore with fluorophore-free media did not show a reduction in the amount of internalized fluorophore.

Example 50: Analysis of delivery to cells by VP22 peptide:
Grow HeLa cells on glass coverslips by incubating at 4°C in Delbecco's Modified Eagle's Media (DMEM) supplemented with 50 μg VP22 peptide-fluorophore chimera (pulse). At this temperature, endocytosis is believed to be completely inhibited. Incubate the cells for two hours at 4°C and then wash with DMEM to remove external VP22-fluorophore. Remove the media and then either process cells for fluorescence microscopy or incubate three more hours at 4°C with DMEM with media changes every hour (chase). The cells that are chased are then processed for fluorescence microscopy. Cells processed for fluorescence microscopy are washed 3x in phosphate-buffered saline (PBS), fixed in PBS + 4% formaldehyde for 20 min, washed 3x in PBS, and coverslips are mounted on slides. The presence of fluorophore is detected by confocal microscopy (Zeiss LSM 510).

Example 51: Analysis of delivery to cells by ANTP peptide:
Grow HeLa cells on glass coverslips by incubating at 4°C in Delbecco's Modified Eagle's Media (DMEM) supplemented with 50 μg ANTP peptide-fluorophore chimera (pulse). At this temperature, endocytosis is believed to be completely inhibited. Incubate the cells for two hours at 4°C and then wash with DMEM to remove external ANTP -fluorophore. Remove the media and then either process cells for fluorescence microscopy or incubate three more hours at 4°C with DMEM with media changes every hour (chase). The cells that are chased are then processed for fluorescence microscopy. Cells processed for fluorescence microscopy are washed 3x in phosphate-buffered saline (PBS), fixed in PBS + 4% formaldehyde for 20 min, washed 3x in PBS, and coverslips are mounted on slides. The presence of fluorophore is detected by confocal microscopy (Zeiss LSM 510).